

(12) **UK Patent Application** (19) **GB** (11) **2 378 899** (13) **A**

(43) Date of A Publication 26.02.2003

(21) Application No 0120654.9

(22) Date of Filing 25.08.2001

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(51) INT CL⁷
A61K 38/36 // A61K 45/00 , A61P 7/06

(52) UK CL (Edition V)
A5B BHA B31X B31Y
U1S S2415

(56) Documents Cited
WO 1996/028548 A1 US 5538861 A
Proceedings of the National Academy of Sciences
USA (2000), vol 97(22), pgs 12260-12265
Journal of Biological Chemistry (1996), vol 271(47),
pgs 30022-30027

(58) Field of Search
UK CL (Edition T) A5B
INT CL⁷ A61K 38/36 45/00, A61P 7/06, C07K 14/745
Other: Online: CAS, BIOSIS, EMBASE, MEDLINE,
SCISEARCH, EPODOC, WPI, PAJ, TXTUS0, TXTUS1,
TXTUS2, TXTUS3, TXTEP1, TXTGB1, TXTWO1

(54) Abstract Title
Treatment of anemia

(57) The present invention relates to a new method for the treatment of anemia based on the administration of the product of growth arrest-specific gene 6 (Gas6), its mutants, variants, active derivatives and the physiological tolerated salts of said Gas6 derivatives or of a group of biologically active substances inducing a GAS6 expression or releasing action or of a group of compounds that activate the Sky, Axl or Mer receptor tyrosine kinases. Furthermore the invention relates to a pharmaceutically effective composition to treat anemia. The composition comprises a pharmaceutically effective amount of a product of growth arrest-specific gene 6 (Gas6), or its mutant, variant, active derivative or the physiological tolerated salts of said Gas6 derivative or a biologically active substance that induces GAS6 expression or GAS6 release or a substance that activates the Sky, Axl or Mer receptor tyrosine kinases.

TREATMENT OF ANEMIA

Field of the invention

The present invention relates to a new method for the treatment of anemia based on the administration of the product of growth arrest-specific gene 6 (Gas6), its mutants, variants, active derivatives and the physiological tolerated salts of these Gas6 derivatives or of a group of biologically active substances inducing a GAS6 expression or releasing action or of a group of compounds that activate the Sky, Axl or Mer receptor tyrosine kinases.

Background

The term *anemia* refers to a reduction below normal in hemoglobin level or red blood cells in the blood. Anemia is a major cause of morbidity and mortality. It may result from a reduced rate of production or increased rate of destruction of red blood cells or their loss from the circulation by bleeding. Although the cause of anemia can be a primary disorder of the production or survival of red blood cells, in many cases it is secondary to diseases of other systems.¹

The mature red blood cell has two main functions. First, it must survive in the circulation for as long as possible, most of the time in vessels smaller than its own diameter. Second, it must both preserve its hemoglobin in state suitable for oxygen transport and adapt the amount of oxygen that is delivered to the needs of the tissues.¹

The process by which erythroid cells are produced is called erythropoiesis. The production of mature red blood cells from pluripotent hematopoietic stem cells requires the coordinated action of different cytokine signaling pathway to assure controlled cell proliferation, survival, differen-

tiation and death. Under normal conditions, the whole process of erythropoiesis results in a red blood production rate such that the red cell mass in the body is kept constant. Erythropoiesis involves a great variety and number of cells at different stages of maturation, starting with the first stem cell progeny committed the erythroid differentiation and ending with the mature circulating red blood cell. Erythropoiesis occurs in distinct stages and anatomic sites during development. Primitive erythropoiesis begins around embryonic day 7 when red blood cells originate in the blood islands of the yolk sac. These cells express embryonic globins and are not dependent upon erythropoietin (EPO).^{2,3} EPO is the major cytokine for definitive and adult erythropoiesis. Definitive erythropoiesis starts at approximately day 10 when the site of red blood cell production shifts from the yolk sac to the fetal liver and is characterised by the production of non-nucleated erythrocytes expressing adult globin genes. At birth, erythroblastic islands in the bone marrow and the red pulp of the spleen become dominant sites of red blood cell production.

Gas6, the product of the growth arrest-specific gene 6 (*gas6*), is a new member of the vitamin K-dependent protein family.^{4,5} Proteins belonging to this family are characterised by post-translational (-carboxylation of certain glutamic acid residues by a carboxylase, using vitamin K as cofactor. The (-carboxyglutamic acid (Gla)-containing module in prothrombin, coagulation factors VII, IX and X, protein C, protein S, protein Z and Gas6 allow these vitamin K-dependent proteins to bind to negatively charged phospholipid membranes.⁶ Gas6 is structurally similar to protein S, but lacks a loop, crucial for the anticoagulant activity of protein S.⁴ The latter is a cofactor for activated protein C, which inactivates the coagulation factors Va and VIIIa.⁷ Genetic deficiency of protein S in man is one of the most severe inherited risk factors for thrombosis.⁸

Apart from a Gla-domain-dependent interaction with phospholipid membranes,⁹ Gas6 also binds as a ligand to the receptor tyrosine kinases Axl (Ark, Ufo, Tyro7), Sky (Rse, Tyro3, Dtk, Etk,

Brt, Tif) and Mer (c-Mer, Eyk, Nyk)¹⁰⁻¹⁴ by its carboxy-terminal globular G domains.¹² It has been implicated in reversible cell growth arrest,^{4,5} survival,¹⁵ proliferation,¹⁵⁻¹⁷ and cell adhesion.^{9,18,19} Mice with a triple deficiency of Axl, Sky and Mer are viable, but have not been reported to suffer spontaneous bleeding or thrombosis.²⁰

We have recently shown that Gas6 deficient (Gas6^{-/-}) mice, generated by homologous recombination, are born at the expected mendelian frequency. Gas6^{+/-} and Gas6^{-/-} mice are viable, fertile, appear normal and show no obvious differences in size, weight or behaviour. No genotypic differences in litter size are observed²¹. These mice are protected against thrombosis because of a platelet dysfunction.²¹

Gas6 expression has been detected in hematopoietic tissue, both in hematopoietic (megakaryocytes and myelomonocytic precursors) and stromal (endothelial cells, fibroblasts, adipocytes) cells.²² Gas6 receptor Axl is expressed in hematopoietic progenitors and bone marrow stromal cells, at low levels in monocytes, and in neoplastic cells of the myeloid lineage.²³ Axl and Sky are detectable at sites of embryonic hematopoiesis.²⁴ However, Gas6 is considered to have no mitogenic effect in the hematopoietic system, and not affecting the proliferative stimuli exerted by other hematopoietic growth factors.²² More recently, monolayers of Gas6 expressing fibroblasts were shown to support the generation of colony-forming units in culture. This hematopoietic support is not vitamin K-dependent and soluble recombinant Gas6 does not substitute for coculturing the hematopoietic progenitors with Gas6 expressing fibroblasts.²⁵

We have now found that Gas6 expression is required for the development of sufficient erythroid reserves in mice. Gas6^{-/-} mice are not anemic but show a reduced level of blood reticulocytes. In addition, they have less cells of the erythroid lineage in adult bone marrow and spleen, and fetal liver at embryonic day E13.5. The decreased number of erythroid cells in the spleen has a slight

but significant impact on the spleen weight as Gas6^{-/-} mice have a smaller spleen than their wild type counterpart. Immunohistochemical analysis of the spleen for Ter-119 (an erythrocytic lineage marker) shows a reduced size of the red pulp (site of red blood cell production) and a lower content in erythroid cells specifically labeled by anti-Ter-119 (an erythrocytic lineage marker) in Gas6^{-/-} mice as compared to Gas6^{+/+} mice. Even fetal liver isolated from Gas6^{-/-} embryos is slightly smaller and contains less erythroid cells than fetal liver from Gas6^{+/+} mice. Gas6^{-/-}, E13.5 embryos are not paler than Gas6^{+/+} embryos, meaning that Gas6^{-/-} embryos do not suffer from severe anemia. Erythroid progenitors are decreased in adult bone marrow and fetal liver at embryonic day E13.5 of Gas6^{-/-} mice as compared to Gas6^{+/+} mice. Finally, by inducing acute hemolytic anemia in mice, we demonstrate that erythropoietic response is markedly reduced in Gas6^{-/-} mice.

Collectively, these data show that Gas6 can be used for the treatment of anemia. It constitutes a new class of promising antianemic drugs.

The present invention will be demonstrated in more detail in the following examples, which are however not intended to limit the scope of the invention.

Summary of the invention

One object of present invention is the use of an activator of the Sky, Axl or Mer receptor tyrosine kinases, for the manufacture of a medicament to treat a patient to prevent, reduce or cure anemia or the use of Gas6, or an analogue, mutant, variant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative for the manufacture of a medicament to treat a patient to prevent, reduce or cure anemia of said patient. Another object can also be the use of biologically active substances inducing a GAS6 expression or releasing action for the manufacture of a medicament

to treat a patient to prevent, reduce or cure anemia of said patient. This medicament can be supplemented with erythropoietin. The anemia can be caused by or associated with various disorders such as chronic disease, chronic renal failure, aplastic anemia, hemolytic anemia, malignancies, endocrine deficiencies or can be caused by a specific treatment such as chemotherapy.

Another object of present invention is the use of Gas6, or an analogue, mutant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative, in combination with erythropoietin for the manufacture of an antianemic drug or an antianemic composition for autologous blood transfusion in a patient. These medicaments can be used to increase the survival rate of the patient suffering of anemia.

Another preferred embodiment of present invention is a method for the treatment, curing or prevention, wherein the patient to be treated receives an effective amount of Gas6, or an analogue, mutant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative, and for a suitable time to prevent, reduce or cure anemia or wherein the patient to be treated receives an effective amount biologically active substances inducing a GAS6 expression or releasing action and for a suitable time to prevent, reduce or cure anemia. This method may further comprise administering of erythropoietin.

Yet another preferred embodiment is an antianemic pharmaceutical composition comprising Gas6, or an analogue, mutant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative. The antianemic pharmaceutical composition can further comprise erythropoietin.

Description

EXAMPLE I: MICE DEFICIENT IN GAS6 (GAS6^{-/-} MICE) HAVE A DECREASED RETICULOCYTE COUNT

Animal experiments were conducted according to the guiding principles of the American Physiological Society and the International Committee on Thrombosis and Haemostasis²⁶

Blood was collected under general anesthesia from retrobulbar plexus of wild type mice (Gas6^{+/+} mice) or mice in which Gas6 expression was abolished by homologous recombination (Gas6^{-/-} mice).²¹ Reticulocyte counts were performed on smears of blood that had been stained with New Methylene Blue according to standard protocol (Sigma R4132). At least 1000 red blood cells were counted in each determination.

The data are represented as mean \pm SEM of n determinations. The significance of differences was determined by unpaired Students'-test. Reticulocytes represented 24.6 ± 4.2 ‰ (n=8) of the circulating red blood cells in Gas6^{+/+} mice and only 13.1 ± 3.1 ‰ (n=8) of the red blood cells in Gas6^{-/-} mice (p<0.001). These data indicate that Gas6 is necessary for maintaining the reticulocyte count within the normal range.

EXAMPLE II: GAS6 DEFICIENT (GAS6^{-/-}) MICE HAVE REDUCED ERYTHROID RESERVES

Single-cell suspensions were obtained from bone marrow and spleen isolated from Gas6^{+/+} and Gas6^{-/-} mice. Ammonium chloride lysis of mature red blood cells was performed. Bone marrow and spleen cells (10^6) were incubated on ice with rat anti-mouse CD16/CD32 to block nonspecific binding to Fc receptors. Cell suspension was then stained with (PE)-conjugated-anti-Ter-119 antibody (PharMingen) for 30 min. at 4°C in 100 μ l phosphate-buffered saline containing 0.2% BSA. Appropriate isotype control antibodies were used. Cell surface expression of Ter-119 was analyzed in a Becton Dickinson FACScan using CellQuest software.

Ter-119 is expressed by cells of the erythroid lineage, from proerythroblast to mature red blood cells. Ter-119 is expressed by 29 ± 3 % (mean \pm SEM, $n=3$) of Gas6^{+/+} bone marrow cells versus 17 ± 3 % (mean \pm SEM, $n=3$) of Gas6^{-/-} bone marrow cells ($p<0.05$). Similarly, Ter-119 is expressed by 6.0 ± 1.3 % (mean \pm SEM, $n=3$) of Gas6^{+/+} spleen cells versus 1.9 ± 0.5 % (mean \pm SEM, $n=3$) of Gas6^{-/-} spleen cells ($p<0.05$).

EXAMPLE III: THE NUMBER OF ERYTHROID PROGENITORS IS DECREASED IN THE BONE MARROW OF MICE DEFICIENT IN GAS6 (GAS6^{-/-} MICE)

In vitro clonogenic assays for progenitor cells allows the study of distinct populations at different stages of development.

Single-cell suspensions were prepared from bone marrow or livers of day 13.5 embryos (E13.5) and counted in the presence of 3% acetic acid to lyse erythrocytes. Cell suspensions were mixed with MethoCult M3434 (StemCell Technologies, Vancouver) as described.²⁷ Cells were plated in 35 mm dishes and cultured at 37°C, 5% CO₂. Colonies including burst-forming units erythroid (BFU-E, early erythroid progenitor) were scored at day 7. For the final progenitor cell colony-forming units erythroid (CFU-E) assay, cells were cultured in MethoCult 3230 containing 0.2 U/ml recombinant murine erythropoietin (R&D Systems) and colonies were scored at day 3.

In Gas6^{+/+} mice the number of BFU-E per 10^5 bone marrow cells was 153 ± 44 (mean \pm SEM, $n=8$), whereas the number of BFU-E per 10^5 bone marrow cells in Gas6^{-/-} mice was 77 ± 35 (mean \pm SEM, $n=8$). Thus, the number of colonies arising from bone marrow early progenitors (BFU-E) was reduced two-fold in Gas6^{-/-} in comparison to Gas6^{+/+} mice ($p<0.05$).

In addition, we studied Gas6^{-/-} mice during fetal development, a time of rapid growth and little reserve capacity of the erythroid lineage. Gas6^{+/+} E13.5 embryos contained $23 \pm 7 \times 10^3$ BFU-E per fetal liver (mean \pm SEM, n=6). In contrast, the number of BFU-E per Gas6^{-/-} fetal liver was $12 \pm 3 \times 10^3$ (mean \pm SEM, n=6). Moreover, Gas6^{-/-} embryos contained fewer final erythroid progenitor cells (colony-forming units erythroid, CFU-E colonies per fetal liver: $34 \pm 8 \times 10^3$ in Gas6^{+/+} embryos, n=6 versus $17 \pm 10 \times 10^3$ in Gas6^{-/-} embryos, n=6, $p < 0.05$).

Thus, erythroid progenitors are decreased in adult bone marrow and fetal liver at embryonic day E13.5 of Gas6^{-/-} mice as compared to Gas6^{+/+} mice.

EXAMPLE IV: IMPAIRED RECOVERY AFTER ACUTE HEMOLYTIC ANEMIA OF GAS6 DEFICIENT MICE (GAS6^{-/-} MICE)

Anemia was induced in Gas6^{+/+} and Gas6^{-/-} mice by intraperitoneal injection (0.5 or 2 mg /10 g body weight) of freshly prepared phenylhydrazine.²⁸ Phenylhydrazine hydrochloride (Sigma P6926) was dissolved in PBS at either 10 or 20 mg/ml and the pH was adjusted to pH 7.4 with NaOH. At day 3 following treatment with low dose of PHZ (two doses of 0.5 mg/10 g, 8 hours apart), Gas6^{-/-} mice had a deeper depression in hematocrit (mean \pm SEM: 29 ± 0.5 %, n=5, $p < 0.001$) than Gas6^{+/+} mice (hematocrit, mean \pm SEM: 36 ± 2 %, n=5). In addition, their spleen (site of red cell production) was weighing less (mean \pm SEM: 142 ± 27 mg n=4, $p < 0.05$) as compared to Gas6^{+/+} mice (mean \pm SEM: 254 ± 28 mg, n=4), indicative of impaired erythropoiesis. Moreover, Gas6^{-/-} mice were more susceptible to hemolysis induced by high dose (2 mg/10 g in one single dose) of PHZ as all Gas6^{-/-} (n=10) succumbed in comparison to 25 % Gas6^{+/+} mice (n=10).

Taken together, these data indicate that the lack of Gas6 expression increase the susceptibility to acute hemolysis.

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CLAIMS

What we claim is:

1. The use of an activator of the Sky, Axl or Mer receptor tyrosine kinases, for the manufacture of a medicament to treat a patient to prevent, reduce or cure anemia.
2. The use of Gas6, or an analogue, mutant, variant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative for the manufacture of a medicament to treat a patient to prevent, reduce or cure anemia of said patient.
3. The use of biologically active substances inducing a GAS6 expression or releasing action for the manufacture of a medicament to treat a patient to prevent reduce or cure anemia of said patient.
4. The medicament of the claims 1,2,3 or 4 comprising erythropoietin.
5. The use according to any of the claims 1 to 4, whereby the anemia results from a condition of chronic disease.
6. The use according to any of the claims 1 to 4, whereby the anemia results from a condition of chronic renal failure.
7. The use according to any of the claims 1 to 4, whereby the anemia is an aplastic anemia.
8. The use according to any of the claims 1 to 4, whereby the anemia is a hemolytic anemia.
9. The use according to any of the claims 1 to 4, whereby the anemia is associated with malignancies.
10. The use according to any of the claims 1 to 4, whereby the anemia is associated with endocrine deficiencies.

11. The use according to any of the claims 1 to 4, whereby the anemia is caused by chemotherapy.
12. The use of Gas6, or an analogue, mutant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative, in combination with erythropoietin for the manufacture of an antianemic drug or an antianemic composition for autologous blood transfusion in a patient.
13. The use according to any of the claims 1 to 12, to increase the survival rate of the patient suffering of anemia.
14. The use according to any one of the preceding claims, wherein the patient to be treated is a mammal, preferable a human being.
15. A method for the treatment, curing or prevention anemia according to each of the previous use claims, wherein the patient to be treated receives an effective amount of Gas6, or an analogue, mutant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative, and for a suitable time to prevent, reduce or cure anemia.
16. A method for the treatment, curing or prevention anemia according to each of the previous use claims, wherein the patient to be treated receives an effective amount biologically active substances inducing a GAS6 expression or releasing action and for a suitable time to prevent, reduce or cure anemia.
17. The method of claim 15 or 16, further comprising administering of erythropoietin.
18. An antianemic pharmaceutical composition comprising Gas6, or an analogue, mutant or derivative thereof or a physiological tolerated salt of said Gas6 derivative.
19. The antianemic pharmaceutical composition of claim 16 further comprising erythropoietin.



INVESTOR IN PEOPLE

Application No: GB 0120654.9
Claims searched: 1-19

Examiner: Richard Swards
Date of search: 26 February 2002

Patents Act 1977 Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.T): A5B

Int Cl (Ed.7): A61K 38/36, 45/00, A61P 7/06, C07K 14/745

Other: Online: CAS, BIOSIS, EMBASE, MEDLINE, SCISEARCH, EPODOC, WPI, PAJ, TXTUS0, TXTUS1, TXTUS2, TXTUS3, TXTEP1, TXTGB1, TXTWO1

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	WO 96/28548 A1 (GENENTECH) see pg 3 ll 18-23, pg 34 ll 4-13, pg 35 ll 13-27, Examples 7, 11 & 12	1-19
A	US 5538861 (AMGEN) see col 2 l 50 - col 3 l 6	
X	Proceedings of the National Academy of Sciences USA (2000), vol 97(22), pgs 12260-12265; Dormady et al; see whole document	1-19
A	Journal of Biological Chemistry (1996), vol 271(47), pgs 30022-30027; Nagata et al; see abstract and Discussion	

X Document indicating lack of novelty or inventive step
Y Document indicating lack of inventive step if combined with one or more other documents of same category.

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